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RAPD Markers to Evaluate Callus Tissue of *Cereus peruvianus* Mill. (Cactaceae) Maintained in Different Growth Regulator Combinations

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RAPD markers were used to detect DNA polymorphisms in callus tissues maintained at different auxin and cytokinin combinations. There is a higher level of genetic variablity in callus tissue maintained with the highest kinetin versus 2, 4-D concentration. Callus tissues subcultured in a 4.0 mg/L 2,4-D and 4.0 mg/L kinetin combination showed high similarity and can be recommended as more suitable sources for industrial procedures of extraction of natural products such as secondary metabolites since extraction protocols can be easily standardized using genetically uniform materials. The higher genetic diversity in callus tissues of C. peruvianus *cultured at 4.0 mg/L 2,4-D and 8.0 mg/L kinetin indicates this tissue as a matrix for in vitro selection of cell lines for higher natural products production. RAPD markers are, therefore, effective tools useful for detecting DNA polymorphism in callus tissue as well as in the DNA identification of callus tissues maintained in different auxin and cytokinin combinations.*

KEY WORDS: RAPD markers; callus tissue; cactus; tissue culture; growth regulators.

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INTRODUCTION

The cactus species *Cereus peruvianus* Mill. has several practical applications. Among the products that can be obtained directly from it are: a viscous gum with potential industrial applications such as in the flocculation of impurities in water for drinking purposes (Nozaki *et al.*, 1993) and in cosmetic preparations (Alvarez *et al.*, 1992); and a pectic compound (pectic acid) that may be used in the preparation of jams, jellies, gums, yogurts, and other products (Alvarez *et al.*, 1995). In order to achieve propagation of *C. peruvianus* cells on an industrial scale, tissue culture-based techniques have been introduced (Machado and Prioli, 1996; Oliveira *et al.*, 1995). Callus tissue is usually the material of choice for *C. peruvianus* plant regeneration (Oliveira *et al.*, 1995) and, as in several other plant species, callus tissue can also be used as a source of natural products such as secondary metabolites (Ellis, 1988; Fowler, 1986) or tissue rich in mucilage (Kokate and Radwan, 1979). In recent studies (Oliveira *et al.*, 1999), *C. peruvianus* callus tissues have been exploited for in vitro selection as an important source of alkaloids. Comparison of alkaloid production from plants and callus tissues indicates higher amounts of alkaloids in callus tissues (Machado and Oliveira, unpublished results).

Callus growing in culture medium containing different combinations of growth regulators showed similar growth and morphology at different cytokinin concentration (Torquato, 1995). Isozyme phenotypes were independent of the cytokinin concentrations tested (Mangolin *et al.*, 1994; Torquato *et al.*, 1995), but the number of proteins with differential expression was higher in callus tissues cultured in the higher cytokinin/auxin proportion (Mangolin *et al.*, 1999). This suggests a differential effect of cytokinin on gene expression in these callus tissues. Isoesterase variants detected in plants regenerated from callus tissue culture of *Cereus peruvianus* suggested the occurrence of somatic crossing-over as a possible mechanism for inducing somaclonal variation (Machado *et al.*, 2000).

Isozyme analysis showed that genetic variation was not observed in long-term callus cultures (Mangolin *et al.*, 1994; Prioli *et al.*, 1995); however, in view of the fact that the process of in vitro culture can result in severe effects on the genetic constitution of plants (Larkin and Scowcroft, 1981), the objective of the present study was to detect DNA polymorphisms in callus tissues maintained at different auxin and cytokinin combinations using RAPD analysis. RAPD markers may reveal a differential effect of cytokinin/auxin proportion on genomic DNA of callus tissues and indicate the auxin and cytokinin combinations that produce more or less genetic variation in *C. peruvianus* callus tissues.

MATERIALS AND METHODS

Callus Tissue Culture

Callus tissues of *C. peruvianus* were subcultured in MS medium (Murashige and Skoog, 1962) containing B5 vitamins (Gamborg *et al.*, 1968), 0.8% agar,

3% sucrose, 4.0 mg/L 2,4-dichlorophenoxyacetic acid (2,4-D) and 4.0 mg/L *N*-(2-furanylmethyl)-1*H*-purine-6 amine (kinetin), or 4.0 mg/L 2,4-D and 8.0 mg/L kinetin, and maintained at 32°C under a photoperiod of 16 h (15 $m^{-2} \cdot s^{-1}$ light intensity). The two callus lines described were induced simultaneously from hypocotyl segments of seedlings, which grew from seeds collected from a single *C. peruvianus* plant (Oliveira *et al.*, 1995). Callus subcultures were performed at 15–20 day intervals, and the callus tissues were, therefore, in culture for the same time. Seven samples of callus tissues subcultured in each one of the two 2,4-D and kinetin combinations were used for DNA extraction.

DNA Extraction and Amplification

For DNA extraction, 1 g of the callus tissue was ground separately to a fine powder with liquid nitrogen in a mortar and pestle. The powder obtained from the samples was transferred to 2 mL Eppendorf tubes and 2.5 volumes of the extraction buffer (10 mM Tris–HCI, pH 8.0, 0.4 M NaCl, 5 mM EDTA, pH 8.0, 0.5% SDS, and 100 μg/mL proteinase K) were added. After homogenization, the samples were incubated at 37°C for 60 min with occasional inversion. Debris was removed by centrifugation at 11,000 rpm for 10 min at room temperature in a microfuge and the supernatant was transferred to a new Eppendorf tube.

The supernatant was extracted three times with an equal volume of phenol:chloroform:isoamyl alcohol (25:24:1) and the DNA was precipitated by addition of one volume of 95% ethanol (Fang *et al.*, 1992). After centrifugation in a microfuge at 11,000 rpm for 10 min at room temperature, the pelleted DNA was dissolved in 500 μL of TE buffer (10 mM Tris–HCl, pH 8.0, 1 mM EDTA) and incubate for 60 min at room temperature with RNase (10 ng/mL; Gibco BRL). The DNA was reprecipitated by addition of NaCl to a final concentration of 2 M and the samples were incubated at −20°C overnight. After incubation one volume of 95% cold ethanol (maintained at −20°C) was added and the samples were agitated gently until homogenized. The samples were centrifuged at 11,000 rpm for 5 min at room temperature and the DNA pellet was washed twice with 100–200 μL 75% cold ethanol. The DNA pellet was air-dried and resuspended in 100–200 μL of TE. The DNA samples were stored at 4°C.

After extraction, DNA concentration and purity were quantified using a Beckman DU-65 spectrophotometer. The A_{260}/A_{280} ratio for the cactus callus DNA samples was around 1.7–1.8, indicating that the samples were relatively pure. The quality of the samples was visualized by electrophoresis in an 0.8% agarose gel (Hoisington *et al.*, 1984). UV quantification was about 140 ng/μL on average for the callus tissues subcultured at the two 2,4-D and kinetin combinations.

The isolated DNA was used as a template in the PCR reactions. The PCR reactions were performed essentially as described by Williams *et al.* (1990), in a total reaction volume of 25 μL containing 25 ng of template DNA, 0.2 μM of each dNTP (Pharmacia), 3.0 mM $MgCl_2$, 2 U Taq-DNA polymerase (Gibco BRL) and

1 × PCR buffer (200 mM Tris–HCl, pH 8.4, 500 mM KCl). Ten-mer primers were used in the amplification reactions. A total of 15 primers (OPB-4, OPB-5, OPB-6, OPB-7, OPB-8, OPB-11, OPB-12, OPB-13, OPB-14, OPB-15, OPB-18, OPB-20, OPC-11, OPC-12, and OPC-13; Operon Technologies Inc. Alameda, CA, U.S.A.) were selected to be used in the amplification reactions using the *C. peruvianus* DNA from seven calli maintained in each 2,4-D and kinetin combination.

The amplifications were performed in duplicate using a Perkin Elmer DNA Thermal Cycler. The PCR conditions were: denaturation for 5 min at 94°C, 45 cyles of 94°C for 1 min, of 35°C for 1 min, and of 72°C for 2 min; with a final 7 min extension at 72°C. Amplification products were separated by electrophoresis on 1.5% agarose gels. Gels were run for 4 h at 3.2 V/cm in TAE buffer, stained with ethidium bromide (0.5 mg/mL) and photographed under UV light (Sambrook *et al.*, 1989).

Bands to be scored were analyzed by comparing the RAPD profiles of different auxin and cytokinin combinations in terms of presence or absence of each DNA fragment in both duplicate reactions. The similarity between strains was calculated using the Jaccard's coefficient and UPGMA cluster analysis was performed using the NTSYS-pc sofware (Rohlf, 1989).

RESULTS AND DISCUSSION

The OPB and OPC primers generated 176 reproducible fragments (Table I) of which 40 and 80 were polymorphic in callus tissues cultured in the 4.0 mg/L

Table I. Polymorphic Fragments in *Cereus peruvianus* Callus Tissues Maintained in Two Culture Media

	2,4-D and kinetin combinations (mg/L)		
Primers	4 × 4[a]	4 × 8[b]	Total bands
OPB-4	3	5	10
OPB-5	1	2	14
OPB-6	1	0	6
OPB-7	3	6	13
OPB-8	1	6	7
OPB-11	4	3	10
OPB-12	3	8	14
OPB-13	3	7	8
OPB-14	7	11	16
OPB-15	3	6	14
OPB-18	3	8	19
OPB-20	3	4	7
OPC-11	2	6	12
OPC-12	2	6	10
OPC-13	1	2	16
Total	40	80	176

[a] Medium containing 2,4-D 4.0 mg/L and kinetin 4.0 mg/L (4 × 4).
[b] Medium containing 2,4,-D 4.0 mg/L and kinetin 8.0 mg/L (4 × 8).

2, 4-D and 4.0 mg/L kinetin, and 4.0 mg/L 2,4-D and 8.0 mg/L kinetin combinations respectively. The number of bands for each primer varied from 6 to 19 with an average of 11.7 fragments per primer. The size of the amplified products ranged from 500 to 5000 bp. Primer OPB-18 generated the highest number of fragments among the primers used, whereas primer OPB-14 had the greatest capacity for discriminating polymorphic fragments in callus tissue cultured at 4.0 × 4.0 mg/L and 4.0 × 8.0 mg/L of 2,4-D and kinetin (Table I). The DNA polymorphism in callus tissues of *C. peruvianus* provides evidence for occurrence of variability produced in in vitro culture since hypocotyl segments of only six seedlings (seeds of the same maternal source) were used as explant to produce the two callus lines (Oliveira *et al.*, 1995).

The observed polymorphic banding patterns reflect the genomic variability present in the callus tissues maintained in culture medium containing 4.0 mg/L 2,4-D and 8.0 mg/L kinetin (Fig. 1) versus the lower kinetin level. The similarity of callus tissues maintained in culture medium containing 4.0 mg/L 2,4-D and 4.0 mg/L kinetin and 4.0 mg/L 2,4-D and 8.0 mg/L kinetin ranged from 77.2 to 94.8% and from 62 to 96.5%, respectively (Table II). The majority of the callus tissues cultured within the 4.0 mg/L 2,4-D and 4.0 mg/L kinetin (C1–C6) were clustered together whereas the majority of the callus tissues cultured within the 4.0 mg/L 2,4-D and 8.0 mg/L kinetin (C8–C14) formed the second group (Fig. 2). Therefore, there is a higher level of genetic variability (similarity from 62 to 96.5%) in callus tissues maintained with the highest kinetin versus 2,4-D concentration. These results support previous observations that altered protein expression occurs during callus growth in the two different 2,4-D and kinetin combinations (Mangolin

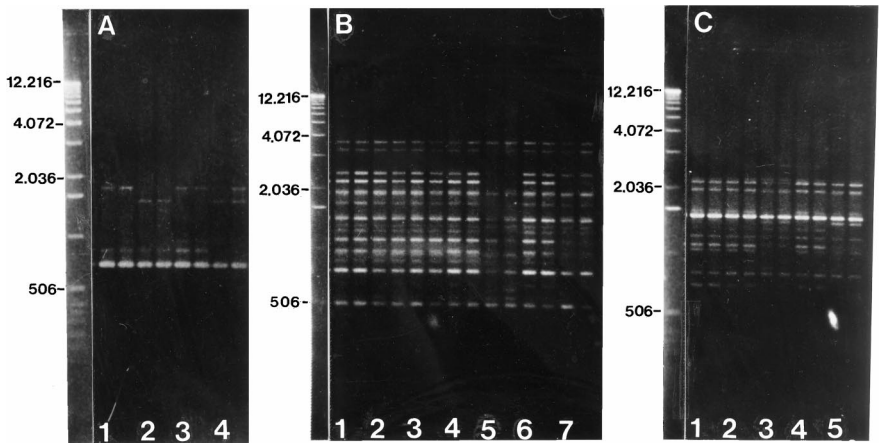

Fig. 1. RAPD profiles from *Cereus peruvianus* callus tissue maintained in culture medium containing 4.0 mg/L 2,4-D and 8.0 mg/L kinetin obtained with primers OPB-8 (A, lanes 1–4), OPB-18 (B, lanes 1–7), and OPB-12 (C, lanes 1–5) from Operon Technologies Inc. (Alameda, CA) on the pairwise samples of genomic DNA. The 1-kb ladder was used as molecular weight marker.

Table II. Jaccard's Coefficient of Similarity of the Two Callus Cultures of *Cereus peruvianus*[a]

C1	1.000												
C2	0.932	1.000											
C3	0.889	0.948	1.000										
C4	0.922	0.925	0.905	1.000									
C5	0.861	0.934	0.942	0.919	1.000								
C6	0.874	0.919	0.900	0.933	0.928	1.000							
C7	0.772	0.826	0.847	0.824	0.859	0.833	1.000						
C8	0.723	0.763	0.795	0.784	0.795	0.794	0.889	1.000					
C9	0.755	0.796	0.829	0.819	0.817	0.828	0.888	0.946	1.000				
C10	0.757	0.787	0.820	0.810	0.796	0.819	0.893	0.952	0.952	1.000			
C11	0.732	0.761	0.794	0.783	0.771	0.792	0.888	0.946	0.933	0.965	1.000		
C12	0.709	0.682	0.671	0.715	0.671	0.691	0.644	0.684	0.703	0.693	0.682	1.000	
C13	0.731	0.759	0.780	0.781	0.791	0.790	0.908	0.941	0.928	0.907	0.915	0.671	1.000
C14	0.620	0.650	0.660	0.637	0.640	0.628	0.675	0.735	0.722	0.734	0.733	0.656	0.732	1.000

[a] C1–C7, cultures maintained at 2,4-D 4.0 mg/L and kinetin 4.0 mg/L. C8–C14, cultures maintained at 2,4-D 4.0 mg/L and kinetin 8.0 mg/L. The coefficients were calculated from RAPD data of 120 polymorphic bands.

et al., 1999). While the 2-D protein pattern of callus tissues cultured within the 4.0 mg/L 2,4-D and 4.0 mg/L kinetin combination was uniform, changes were detected due to the presence or absence of five polypeptides (Mr 49.9, Mr 47.8, Mr 38.2, Mr 28.0, and Mr 25.3) in callus tissues cultured in 4.0 mg/L 2,4-D and 8.0 mg/L kinetin (Mangolin *et al.*, 1999).

Our results show that RAPD markers are effective tools for indicating differential effects of kinetin concentrations in genomic DNA of callus and for

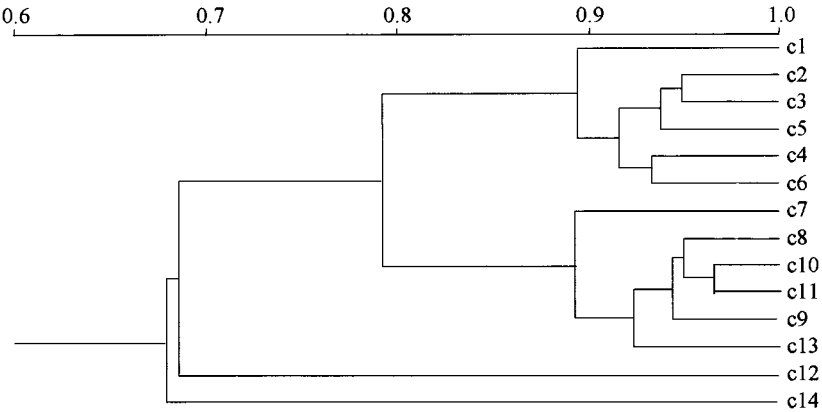

Fig. 2. Dendrogram representing the relationship between the two callus cultures of *Cereus peruvianus* maintained in 2,4-D 4.0 mg/L and kinetin 4.0 mg/L (C1–C7), and 2,4-D 4.0 mg/L and kinetin 8.0 mg/L (C8–C14) combinations based on UPGMA cluster analysis of the RAPD profiles derived from 15 primers using Jaccard's similarity coefficient.

distinguishing among individual callus tissues. This evidence confirmed our theory that RAPD markers seem to reveal more variation than isozymes since the isozyme analyses reflect only variations in constitutive genes that are subject to environmental and developmental alterations (Jorge *et al.*, 1997; Mangolin *et al.*, 1997; Torquato *et al.*, 1995), whereas RAPD analyses provide random access to constitutive and nonconstitutive genes of the genome.

The present study shows also that the callus tissues subcultured at the 4.0 mg/L 2,4-D, 4.0 mg/L kinetin combination showing high similarity are a more suitable source for industrial procedures of extraction of the alkaloids since extraction protocols can be easily standardized using genetically uniform materials. On the other hand, the callus tissues subcultured at the 4.0 mg/L 2,4-D, 8.0 mg/L kinetin combination showing a higher level of genetic diversity are recommended as matrices for in vitro selection of cell lines showing a higher alkaloid production when subcultured in medium supplemented with alkaloid precursors.

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